

## OSMOTIC AND IONIC REGULATION IN *LUMBRICUS TERRESTRIS* L.<sup>1</sup>

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Earthworms normally live in moist soil but during rainy seasons they are exposed to an aquatic environment. In the laboratory they survive several months in freshwater of the appropriate ionic composition (Roots, 1956). Because of the difficulties in controlling soil conditions it is of advantage to study the basic features of osmotic regulation under freshwater conditions. This is particularly important in the analysis of ionic regulation where rigorous control of the external bath is essential. In fact most of the published information on hydromineral metabolism in *Lumbricus* is on animals in freshwater (Adolph, 1927; Maluf, 1939, 1940; Wolf, 1940; Bahl, 1945; Ramsay, 1949ab; Kamemoto, Spaulding and Keister, 1962; van Brink and Rietsema, 1949). While these studies have established many important features of osmotic regulation in *Lumbricus*, the mechanisms of osmotic and, particularly, ionic regulation in these animals are poorly understood.

For this reason we undertook a systematic analysis of hydromineral metabolism in the earthworm living under freshwater conditions. Secondly, we analyzed some of the adjustments in hydromineral balance these animals undergo when transferred from soil to freshwater. Finally we sought to obtain some preliminary information on the control of the various mechanisms involved in osmotic and ionic regulation in these animals.

### METHODS

#### 1. *Animals*

*Lumbricus terrestris* weighing 1 to 10 g (mean 3 g) were obtained from a local bait dealer and kept in a leaf mulch soil at  $14 \pm 1^\circ \text{C}$ , on a 14 hr photoperiod. Water was added to the soil periodically but soil moisture was not rigorously controlled. Most animals were equilibrated in artificial pond water (0.5 mM NaCl, 0.05 mM KCl, 0.40 mM  $\text{CaCl}_2$  and 0.20 mM  $\text{NaHCO}_3$ ) for at least one week prior to use. The bath was continuously aerated.

#### 2. *Coelom, crop and rectal fluid*

Coelomic fluid (CF) was collected by inserting a small, pointed capillary tube through the body wall into the coelom of worms immobilized by packing in ice.

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The crop was exposed by cutting through the skin. Adherent CF was removed by blotting and a pointed capillary tube inserted into the crop and the fluid collected. Rectal fluid was collected by inserting a capillary tube through the anus. Particulate matter was removed by centrifugation.

### 3. Chemical analyses

Sodium and potassium concentrations in diluted CF, rectal and crop fluid were determined by flame photometry; chloride by electrometric titration. Ammonium was determined colorimetrically by direct nesslerization. Total solute was determined on undiluted samples using a Mechrolab vapor pressure osmometer.

Total  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  contents of earthworms were also determined. The animals were dried to a constant weight at  $105^\circ\text{C}$ . For  $\text{Na}^+$  and  $\text{K}^+$  determination the carcasses were digested with hot ( $40\text{--}50^\circ\text{C}$ ) concentrated  $\text{HNO}_3$ . The digest was diluted for analysis by flame photometry. For  $\text{Cl}^-$ , the dried animals were ground to a powder and extracted with distilled water for 12 to 24 hr at  $3^\circ\text{C}$ . Particulate matter was removed by centrifugation or filtration and the water extract was analyzed for  $\text{Cl}^-$  by electrometric titration.

### 4. Coelomic fluid clearance

Clearance from the CF of C-14-inulin, high molecular weight dextran, and glycine was measured on separate animals. The labeled compound diluted in an iso-osmotic saline solution was injected into the coelomic cavity; each animal was placed in pond water (PW) and left one hour to allow distribution of the isotope in the CF (separate experiments indicated distribution was complete within two hours). Each animal was then rinsed, weighed and transferred to 10 ml of PW. The containers with the animals were gently shaken at  $15 \pm 1^\circ\text{C}$ . Samples were removed from the bath at one hour intervals for 4 hr and assayed for radioactivity by liquid scintillation methods. At the end of the experiment a CF sample was collected and counted. The rate of counts appearing in the bath is the product of the urine radioactivity and the volume excreted. From the bath and CF radioactivity the clearance of each substance was determined. Since the total radioactivity loss from the CF was less than 10 per cent, the CF was considered constant. The technique assumes that drinking is negligible in these animals (see below).

### 5. Ion fluxes

Sodium and chloride influx was measured with Na-22 or Cl-36, respectively. The radioactive isotope was added to a bath of known volume and ionic composition. Quantitative samples were withdrawn from the bath at specified intervals, placed in a planchet, evaporated to dryness, and assayed for radioactivity. A separate sample was assayed for  $\text{Na}^+$  by flame photometry or  $\text{Cl}^-$  by electrometric titration. The influx was calculated from the rate of diminution of radioactivity in the bath (see Jørgensen, Levi and Ussing, 1946). The net flux was obtained from the chemical analysis. The efflux then was computed by difference (net flux = influx - efflux).

### 6. Transepithelial electrical potentials

The transepithelial electrical potential (TEP) generated by earthworms *in vivo* was measured as described by Dietz, Kirschner and Porter (1967). The basic bathing solution was 1 mM  $K_2SO_4$  to obtain a conducting medium. Solutions were added to the bath to give the required ionic composition and concentration.

### 7. Statistical treatment

All values for populations of animals are presented as the mean  $\pm$  one standard error of the mean. Differences between means were analyzed by the student "t" test and considered significant if  $P < 0.05$ .

## RESULTS

### 1. Effect of ionic composition of the bath on CF composition

Groups of six to eight worms were kept in baths of varying concentration of NaCl for 10 to 13 days whereupon the ionic composition of their CF was determined. The results are shown in Figure 1. Earthworms hyper-regulate with respect to  $Na^+$  and total solute in solutions below 78 mM NaCl. Above this the CF remains slightly hyper-osmotic but the  $Na^+$  concentration is equal to the bath. Chloride is hyper-regulated in solutions below 40 mM NaCl but hypo-regulated above this. Over the entire range,  $Na^+$  and  $Cl^-$  account for most of the solute in CF. There is a slight increase in the total solute above that contributed by  $Na^+$  and  $Cl^-$  in the animals acclimated to 126 and 150 mM NaCl. The source is unknown. Potassium concentration is about 3 mM/l and remained constant.

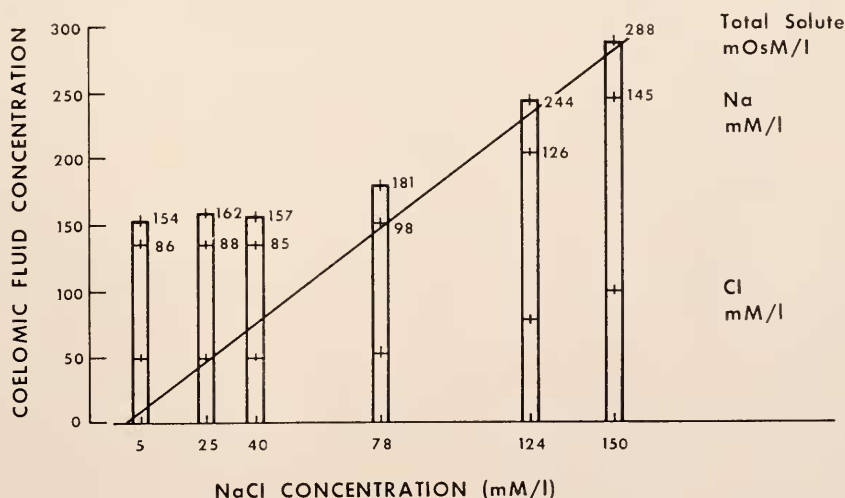


FIGURE 1. Coelomic fluid composition of *Lumbricus terrestris* acclimated 10 to 13 days in different NaCl solutions. Sodium is added to chloride in each column. The number adjacent to each bar is the mean concentration of the ion indicated ( $N = 8$ ). Vertical lines indicate  $\pm$  one S.E. The diagonal line represents iso-osmoticity.

Although survival was high in solutions containing at least 5 mM NaCl we found that earthworms do not survive more than two weeks in dilute NaCl solutions ( $< 1.0$  mM) or distilled water. Yet they survived indefinitely in an artificial pond water containing a dilute mixture of ions.

## 2. Adjustment to PW

Upon transfer from soil to PW earthworms gain weight for about two days as shown in Figure 2. A steady state is then reached at a weight of 115% of the weight in soil ( $B_0$ ). This increase in weight reflects the net accumulation of water which initially causes a dilution of the solute in the CF. The reduction is transitory, however, because after seven days in PW concentrations of ions in the CF are very similar to those of soil animals (Table I) and this does not involve a loss of water (Fig. 2). Either net quantities of ions are accumulated from the bath or ions are

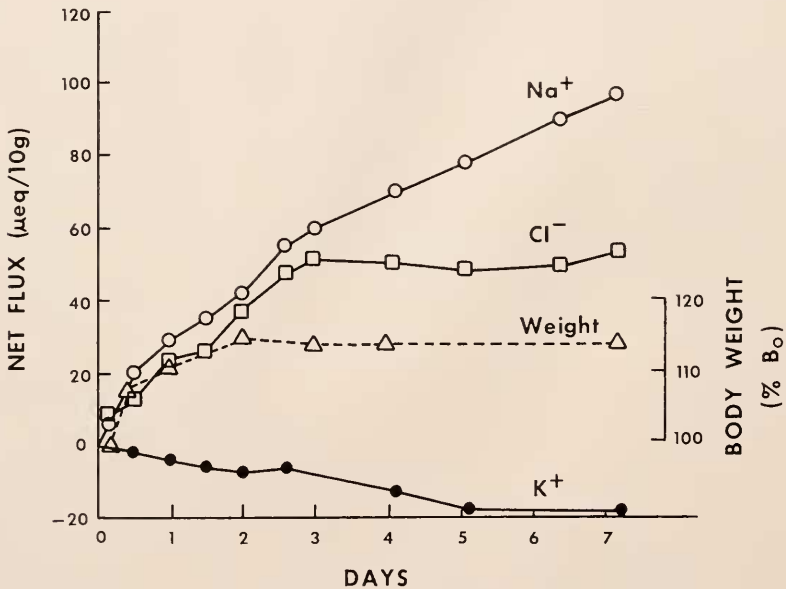


FIGURE 2. Net exchange of ions and water for worms transferred from soil to 50 ml of PW. Each point is based on the mean of eight worms. The initial body weight was  $2.7 \pm 0.4$  g.

mobilized from stores within the animal. Evidence for the former hypothesis is shown in Figure 2. Animals were transferred individually from soil to a limited volume of PW. Changes in bath concentrations of  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  were followed and the net exchanges calculated. A net uptake of  $\text{Na}^+$  and  $\text{Cl}^-$  which begins almost immediately is apparent. In the experiment depicted, the animals came into a steady state with respect to  $\text{Cl}^-$  in three days but continued to accumulate  $\text{Na}^+$  throughout the experiment. In other experiments animals did approach a Na-steady state in seven days. It is particularly noteworthy that the uptake of  $\text{Cl}^-$  does

TABLE I  
*Ionic composition and concentrations of ions in coelomic fluid (CF) of  
 Lumbricus acclimated to soil or pond water*

Measurement	Units	Soil	Pond water
Water content	ml/10 g†	8.4 ± 0.1 (18)	8.8 ± 0.0* (22)
Total Na <sup>+</sup>	μeq/10 g	235 ± 3 (8)	373 ± 15* (12)
Total K <sup>+</sup>	μeq/10 g	345 ± 15 (8)	365 ± 15 (12)
Total Cl <sup>-</sup>	μeq/10 g	172 ± 8 (8)	174 ± 13 (12)
CF Na <sup>+</sup>	meq/l	71 ± 2 (14)	75 ± 1 (24)
CF K <sup>+</sup>	meq/l	4 ± 0 (14)	3 ± 0 (24)
CF Cl <sup>-</sup>	meq/l	48 ± 1 (14)	47 ± 1 (24)
CF Total solute	mOsm/l	154 ± 2 (10)	159 ± 2 (15)

† Wet weight.

\* Significantly different from soil animals ( $P < 0.05$ ).

not equal that of Na<sup>+</sup>, and the time courses of uptake are not necessarily similar. Potassium is continually lost.

These data show that earthworms have a delicately controlled iono-regulatory system. Presumably dilution of the body fluids upon placement in PW activates the iono-regulatory systems. The nature of this activation will be explored in a subsequent section (control of ionic regulation). However, first we shall examine water and ion exchanges in animals acclimated to PW.

### 3. Water exchanges

Earthworms in PW can take up water across the gastro-intestinal tract and/or the skin; they can lose water by nephridial excretion or rectal excretion. In PW, worms open their mouths periodically and they will also feed on particulate matter in the water. To determine if fluid is ingested, worms were placed in PW containing C-14-inulin (0.1 μc) and left for nine days, whereupon samples of the crop, midgut and CF were collected and assayed for radioactivity.

The crop fluid radioactivity ranged between 29 and 69% of the bath radioactivity. The midgut fluid was between 39 and 69% of the bath activity but the CF did not exceed 5% of the bath activity. Apparently these animals drink. The drinking rate was estimated from a water extract of dried worms which had been in PW with C-14-inulin for 10 hr. The mean rate for five animals was 4.2 ± 1.0 μl/10 g-hr.

Measurement of the rate of urine flow is difficult, but a maximal estimate can be obtained by measuring clearance rates of certain substances from the CF. The material must not be stored or metabolized, its entry into the nephridia must be unrestricted and there can be no reabsorption from the nephridia. If the presumptive urine entering the nephridia is coelomic fluid entering via the nephrostome, then all substances satisfying the assumptions should give the same clearance value. We checked C-14 labeled glycine, inulin and a high molecular weight dextran expecting that the first would be reabsorbed while the last two would meet the above mentioned requirements. Table II shows that PW-acclimated worms clear inulin and dextran at similar rates and glycine clearance is only about  $\frac{1}{7}$  as great.

TABLE II

*The rates of clearance of inulin, dextran and glycine from the coelomic fluid of the earthworm, Lumbricus terrestris, immersed in pond water*

Substance	Clearance ( $\mu\text{l}/10\text{ g-hr}$ )
PW acclimated	
Inulin	70 $\pm$ 11 (7)
Dextran	76 $\pm$ 19 (5)
Glycine	10 $\pm$ 4 (5)
Soil acclimated	
Inulin	10 $\pm$ 2 (4)
Inulin	83 $\pm$ 19 (6)

Soil-acclimated animals, placed in PW, responded variably. Six out of ten animals had similar clearance rates comparable to PW animals while in four the rates were only  $\frac{1}{8}$  as fast. The reason for this difference is not known but it suggests that animals in soil have a low clearance rate which is elevated in response to exposure to PW. Individuals may differ in the time required to elicit the response even though water is entering osmotically.

Curiously, the clearance of inulin and dextran remained unchanged when PW-acclimated worms were placed in iso-osmotic sucrose solution for six hr. Apparently the rate of osmotic uptake of water does not immediately affect the rate of clearance of CF.

No urine analyses were performed so the amount of water absorbed by the nephridia could not be determined. It is likely that some reabsorption occurs so that the rate of urine flow would be less than the clearance rate of inulin (75  $\mu\text{l}/10\text{ g-hr}$ ).

Earthworms in PW void a fluid from the anus. We have not measured the rate of elimination of this rectal fluid but Wolf (1940) estimated a rate of 22  $\mu\text{l}/10\text{ g-hr}$ . Animals injected (CF) with C-14-inulin or C-14-dextran did not void either compound through the anus. The source of the rectal fluid is not known.

#### 4. Sodium and chloride balance

Worms acclimated to PW maintain a steady state with respect to  $\text{Na}^+$  and  $\text{Cl}^-$ . The unidirectional fluxes are given in Table III. Chloride influx is greater than  $\text{Na}^+$  influx possibly because  $[\text{Cl}^-]$  is greater than  $[\text{Na}^+]$  in PW (see below).

Solutes may be lost through the gastro-intestinal tract, the nephridia, or across the skin. Crop fluid is remarkably similar to CF in  $[\text{Na}^+]$ ,  $[\text{K}^+]$ , and total solute, but considerably lower in  $[\text{Cl}^-]$  (Table IV). Since drinking is minimal in PW, most of the crop fluid must be derived from the body fluids. We never observed regurgitation of fluid through the mouth so that the crop fluid must pass posteriorly through the intestine and be subjected to alteration. Rectal fluid is more dilute than CF or crop fluid (Tables II and IV) and approaches published values for earthworm urine (Bahl, 1945; Ramsay, 1949 ab). The mechanism of formation

TABLE III

*Mean Na<sup>+</sup> and Cl<sup>-</sup>-unidirectional fluxes in PW-acclimated Lumbricus terrestris.  
The average weight of the worms was 4.4 ± 0.3 g.*

Ion	N	Flux (μeq/10 g-hr)	
		M <sub>i</sub>	M <sub>o</sub>
Na <sup>+</sup>	15	0.32 ± 0.04	0.37 ± 0.07
Cl <sup>-</sup>	15	0.88 ± 0.10	0.90 ± 0.14

of crop fluid and its subsequent alteration remain unknown. It would seem that the possibility of a filtration-reabsorption system comparable to that found in excretory organs in other fresh-water animals might be worth exploring (see discussion). If Wolf's (1940) estimate of the rate of rectal fluid elimination (0.022 ml/10 g-hr) is correct than about 36% of the Na<sup>+</sup> and 32% of the total Cl<sup>-</sup> is lost via this route. The rest must be lost through the nephridia or the skin.

We have not been able to obtain a direct estimate of the nephridial component of the efflux. However, it is possible to establish some limits. The clearance of inulin (75 μl/10 g-hr) gives a maximal estimate of the rate of urine flow. The urine concentration of Na<sup>+</sup> and Cl<sup>-</sup> was not measured but if there were no loss of these ions across the skin the urine concentration could not exceed 3.2 μeq/ml for Na<sup>+</sup> and 8.1 μeq/ml for Cl<sup>-</sup>. Published values for urine [Cl<sup>-</sup>] are about 3.5 μeq/ml (Bahl, 1945; Ramsay, 1949ab) so that a maximum of 0.26 μeq Cl/10 g-hr, or 29% of the Cl<sup>-</sup> efflux, would be through the nephridia. This means that at least 0.35 μeq/10 g-hr or 39% of the Cl efflux is across the skin. No information is available on urine Na<sup>+</sup> concentration.

To demonstrate active transport of an ion, it must be shown that the ion is accumulated against an electrochemical gradient. Transepithelial electrical potentials (TEP) were measured on worms acclimated to PW and exposed to a range of NaCl or Na<sub>2</sub>SO<sub>4</sub> concentrations. The TEP is a logarithmic function of the Na<sup>+</sup> concentration and independent of the anion. At low [Na<sup>+</sup>] (0.2 meq/l) the CF is negative to the bath (-20 to -40 mV). When the bath [Na<sup>+</sup>] is greater than 20 mM the inside became positive. The TEP values of worms acclimated to PW and measured in PW were between -30 and -12 mV.

TABLE IV

*Ionic composition of crop fluid and rectal fluid of Lumbricus acclimated to pond water*

Ion	Concentration (m-Moles/l)	
	Crop	Rectal fluid
Na <sup>+</sup>	62 ± 3 (14)	6 ± 2 (7)
K <sup>+</sup>	4 ± 0 (10)	3 ± 0 (3)
Cl <sup>-</sup>	2 ± 0 (14)	13 ± 5 (9)
N	—	14 ± 2 (7)
Total solute*	153 ± 4 (16)	36 ± 10 (6)

\* mOsm/l.

If the inward and outward permeability coefficients for an ion are the same, then the passive diffusion of that ion should obey the flux-ratio equation (Ussing, 1949):

$$M_i/M_o = (c_o/c_i) \exp(zFE/RT) \quad (1)$$

where the subscripts  $i$  and  $o$  refer to the inside and outside compartments,  $M$  is the flux in  $\mu\text{eq}/\text{cm}^2\text{-hr}$ ;  $c$  is the ionic concentration in  $\mu\text{eq}/\text{cm}^3$ ;  $z$  is the valence of the ion;  $F$  is the Faraday, 96,500 joules/volt mole;  $E$  is the potential difference across the membrane in volts;  $R$  is the gas constant 8.3 joule/degree mole and  $T$  is the absolute temperature. In a steady state the flux ratio ( $M_i/M_o$ ) is equal to one. If an ion is passively distributed then a calculation of the expected flux ratio should equal the observed flux ratio. For animals in PW the calculated flux ratios would be 0.004 and 0.059 for  $\text{Na}^+$  and  $\text{Cl}^-$ , respectively, assuming that the TEP is 20 mV (inside negative). Transport of both  $\text{Na}^+$  and  $\text{Cl}^-$  is thermodynamically active.

The integument is the primary site of  $\text{Na}^+$  transport. This was determined by measuring the influx of Na-22 on seven worms which had their anterior and posterior ends ligated with heavy cotton string. The influx over the 10 hr interval was  $1.76 \pm 0.16 \mu\text{eq Na}^+/10 \text{ g-hr}$  with a net uptake of  $0.12 \pm 0.12 \mu\text{eq Na}^+/10 \text{ g-hr}$ . Handling stress is probably responsible for the high influx.

To further characterize the  $\text{Na}^+$  transport mechanism, experiments were performed measuring the influx of  $\text{Na}^+$  over a range of external  $\text{Na}^+$  concentrations. The basic solution for these studies was PW with different amounts of NaCl added or deleted. Worms were acclimated to PW prior to the flux measurements shown in Figure 3. The rate of  $\text{Na}^+$  transport is dependent on bath  $[\text{Na}^+]$  at low concen-

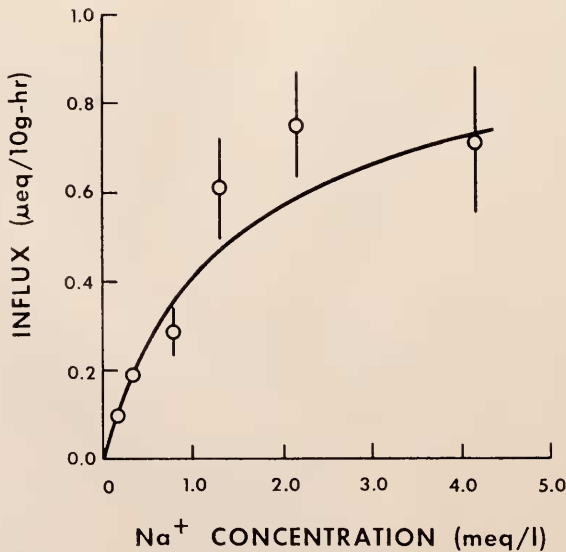


FIGURE 3. The effect of external  $\text{Na}^+$  concentration on the influx of  $\text{Na}^+$  into PW-acclimated worms. Each point represents a mean of at least six animals. Vertical lines represent  $\pm$  one S.E. The mean weight of the animals was  $4.3 \pm 0.2 \text{ g}$  (56).



trations and Michaelis-Menten kinetics apply. The maximum influx ( $V_{\max}$ ) for PW-acclimated worms is  $0.96 \mu\text{eq}/10 \text{ g-hr}$ . The  $\text{Na}^+$  concentration at which the influx is half  $V_{\max}$ , or  $K_s$ , is  $1.3 \text{ mM Na}/1$ .

### 5. Control of $\text{Na}^+$ and $\text{Cl}^-$ balance

Experiments described in a previous section show that the dilution of the body fluids following transfer of worms from soil to PW is due to a net uptake and retention of water. Eventually  $\text{Na}^+$  and  $\text{Cl}^-$  were accumulated restoring their CF concentrations to levels comparable to soil animals. The kinetics of this adjustment were analyzed by following  $\text{Na}^+$  and  $\text{Cl}^-$  fluxes during various parts of the adjustment period. The results are shown in Table V.

TABLE V

*$\text{Na}^+$  and  $\text{Cl}^-$  unidirectional fluxes in worms transferred from soil to PW. The animals weighed  $3.4 \pm 0.1 \text{ g}$  (48). Each value represents a mean of 8 animals*

Hours in PW	Flux ( $\mu\text{eq}/10 \text{ g-hr}$ )			
	$\text{Na}^+$		$\text{Cl}^-$	
	$M_i$	$M_0$	$M_i$	$M_0$
0-24	$1.34 \pm 0.15$	$0.21 \pm 0.05$	$2.94 \pm 0.23$	$1.06 \pm 0.14$
36-72	$0.84 \pm 0.09$	$0.51 \pm 0.06$	$1.87 \pm 0.24$	$1.60 \pm 0.20$
90-130	$0.52 \pm 0.14$	$0.51 \pm 0.14$	$0.73 \pm 0.15$	$0.86 \pm 0.11$

The net uptake of  $\text{Na}^+$  and  $\text{Cl}^-$  is most pronounced during the first interval although some is accumulated during the second. By the third interval a steady state is restored. The net uptake results primarily from an elevated influx of each ion, although in the case of  $\text{Na}^+$  there is also a reduction in the efflux during the first period.

When worms enter PW there is both an increase in water content and a dilution of the body fluids either one of which could serve as the stimulus for the responses indicated above. By exposing PW-acclimated worms to distilled water (DW), it is possible to reduce the solute concentration of the CF without affecting water content. *Lumbricus* will not tolerate prolonged exposure to DW (50% mortality in one week), however, they will survive three or four days. During this period they suffer a net loss of  $\text{Na}^+$  which is highest during the first four hr ( $0.35$  to  $2.39 \mu\text{eq Na}^+/10 \text{ g-hr}$ ) and then stabilizes to a mean value of  $0.28 \pm 0.05 \mu\text{eq}/10 \text{ g-hr}$  between 4 and 72 hr. Even though the bath volume was limited (50 ml) these worms did not come into a steady state with respect to  $\text{Na}^+$ . We did observe that  $\text{Na}^+$  loss in the presence of  $\text{Ca}^{++}$  in the bath is 30% lower than the loss to distilled water.

The loss of ions experienced during exposure to distilled water is reflected by a reduction in their concentration in the CF. Thus after three days [ $\text{Na}^+$ ] was reduced 19% and [ $\text{Cl}^-$ ] 39% relative to PW-acclimated animals. The animals do not gain weight in DW.

When returned to PW, salt-depleted worms experience a net uptake of  $\text{Na}^+$  and  $\text{Cl}^-$  which can be attributed primarily to a marked increase in the influx of each ion (Table VI). It appears that the reduction in CF ion concentration stimulates the transport systems.

TABLE VI

Mean  $\text{Na}^+$  and  $\text{Cl}^-$  unidirectional fluxes for salt-depleted worms placed in PW. Each value represents an average of 4 animals measured over 22 hours. The animals weighed  $2.9 \pm 0.3$  g

Ion	N	Flux ( $\mu\text{eq}/10$ g-hr)	
		$M_i$	$M_o$
$\text{Na}^+$	4	$2.83 \pm 1.01$	$1.35 \pm 0.46$
$\text{Cl}^-$	4	$3.88 \pm 0.86$	$0.78 \pm 0.68$

### 6. Independence of $\text{Na}^+$ and $\text{Cl}^-$ transporting systems

Since salt depletion stimulates both  $\text{Na}^+$  and  $\text{Cl}^-$  transport systems an experiment was performed to determine if there is a dependency between them. Six worms, salt depleted for four days, were placed in individual containers having either 0.35 mM  $\text{Na}_2\text{SO}_4$  or 1.30 mM KCl and the  $\text{Na}^+$  and  $\text{Cl}^-$  flux, respectively, was measured over 21 hr (Table VII). Clearly sodium and chloride can be transported independently although at a lower rate than that measured from PW.

TABLE VII

Mean  $\text{Na}^+$  and  $\text{Cl}^-$  unidirectional fluxes for salt-depleted worms exposed to 0.3 mM  $\text{Na}_2\text{SO}_4$  or 1.25 mM KCl. Each value represents an average of 3 animals measured over 21 hours. The animals weighed  $4.6 \pm 0.7$  g

Ion	N	Flux ( $\mu\text{eq}/10$ g-hr)	
		$M_i$	$M_o$
$\text{Na}^+$	3	$0.58 \pm 0.05$	$0.36 \pm 0.01$
$\text{Cl}^-$	3	$1.69 \pm 0.60$	$0.67 \pm 0.32$

Potassium is not accumulated and separate studies revealed that  $^{35}\text{SO}_4^{2-}$  is not taken up from the bath. In order to preserve electroneutrality the net uptake of  $\text{Na}^+$  from  $\text{Na}_2\text{SO}_4$  and of  $\text{Cl}^-$  from KCl must involve exchange with an endogenous ion of like charge. Either  $\text{NH}_4^+$  or  $\text{H}^+$  could be exchanged for  $\text{Na}^+$  and  $\text{HCO}_3^-$  or  $\text{OH}^-$  for  $\text{Cl}^-$ . All of these are metabolic products which are produced at rates commensurate with such an exchange system.

### DISCUSSION

Survival of earthworms in an aquatic medium is dependent on the ionic composition of the bath. They do not survive more than one or two weeks in distilled water or 1.0 mM NaCl. They will survive in more concentrated solutions of

NaCl (5 mM or greater) or in an artificial pond water. The basis for these observations has not been determined. Calcium probably has a direct effect on permeability of exposed epithelia. Sodium loss to dilute  $\text{CaCl}_2$  solutions is slower than to distilled water. A similar observation has been made on the ammocoetes larva by Morris and Bull (1968). Most of our experiments were performed on animals in an artificial pond water containing  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{++}$ ,  $\text{HCO}_3^-$  and  $\text{Cl}^-$  (see above). We have kept worms several months in this medium. The ionic concentrations in PW are similar to soil water (Davson, 1964).

Animals acclimated several days in PW maintain a constant body weight despite a large osmotic gradient favoring the uptake of water. The mechanisms for maintaining water balance are in most respects similar to those of other fresh-water animals (Krogh, 1939; Potts and Parry, 1964). These include a relatively low permeability to water, low drinking rate and the capacity to excrete a urine hypo-osmotic to the body fluids. In addition the gastro-intestinal tract of earthworms may be involved in the elimination of water.

The rate of urine production was not estimated directly, but if our assumptions relating to the clearance of inulin and high molecular weight dextran are valid, the rate of urine production cannot exceed the rate of clearance of these compounds, or about  $75 \mu\text{l}/10 \text{ g-hr}$ . The rate of excretion of rectal fluid has been estimated by Wolf (1940) at  $22 \mu\text{l}/10 \text{ g-hr}$ . Thus the rate of elimination of water is less than  $100 \mu\text{l}/10 \text{ g-hr}$  (24% of body weight/day).

In the steady state this value equals the osmotic uptake of water and is comparable to what is found in a variety of fresh-water animals (Krogh, 1939, Potts and Parry, 1964). This is lower than the value of  $250 \mu\text{l}/10 \text{ g-hr}$  estimated by Wolf (1940). The discrepancy can probably be attributed to differences in technique since he blotted the animals and this may have increased their permeability to water.

There are two sites of entry of water, the integument and the gut. Maluf (1939) has shown that ligation of anterior and posterior ends of worms does not prevent the net uptake of water when worms are transferred from soil to water. Earthworms are like most fresh-water animals in that drinking is minimal. By placing animals in a bath containing C-14-inulin we have found that the volume of bath passing into the digestive tract is about  $4 \mu\text{l}/10 \text{ g-hr}$ . If all of this were absorbed it would account for about 4% of the water taken up by the worms. Obviously the major route of uptake of water is across the body surface. We have no accurate data of the area of a 10 g worm but  $50 \text{ cm}^2$  would seem reasonable (surface area =  $10 \text{ Wt}^{2/3}$ ). The permeability constant for water then would be about  $2 \mu/\text{sec}$ . This is slightly higher than most fresh-water animals which usually range between 1 and  $0.1 \mu/\text{sec}$  (Potts and Parry, 1964), but comparable to others such as the frog ( $4 \mu/\text{sec}$ ).

The formation of urine by nephridia involves movement of CF through the nephrostome by action of cilia. This presumptive urine is then modified by reabsorption and possibly secretion. Boroffka (1965) has shown that dilution of urine occurs primarily in the distal portion of the nephridia by active reabsorption of  $\text{Na}^+$ . The tubule must be relatively impermeable to water.

Rectal fluid is also quite dilute whereas crop fluid is virtually iso-osmotic with CF. The origin of crop fluid remains unknown however conceivably it is a

filtrate of blood. Blood is quite similar to CF (Kamemoto *et al.*, 1962). Thus blood entering the posterior esophagus (region of the calciferous glands) could be filtered into the G-I tract. The presence of large blood sinuses separated from the lumen of the esophagus by a thin layer of cells is structurally suited for filtration (Semal, 1959; Laverack, 1963). We were unable to demonstrate the filtration of inulin into the gut. Perhaps the molecule is too large. The  $[\text{Cl}^-]$  of crop fluid is very low (Table IV) probably because there is an exchange of  $\text{Cl}^-$  for  $\text{HCO}_3^-$  between blood and the gut. In the presence of  $\text{Ca}^{++}$  some of the  $\text{HCO}_3^-$  would form  $\text{CaCO}_3$  (Robertson, 1936).

As the fluid passes posteriorly solute could be reabsorbed to form the hypo-osmotic rectal fluid. Some of the  $\text{Na}^+$  removed from the gut may be in exchange for  $\text{NH}_3$  (Table IV; Tillinghast, 1967). This hypothesis is highly speculative but would appear to be a more satisfactory explanation than an alternative which would be transport of water from blood or CF to the gut to produce a hypo-osmotic gut fluid.

Living in PW without food, earthworms hyper-regulate  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{K}^+$ , and total solute. The turnover time of  $\text{Na}^+$  and  $\text{Cl}^-$  would be 49 and 8 days, respectively. Ions are lost across the body surface by diffusion and excretion through the gut and urine. For  $\text{Cl}^-$  the approximate contribution of each of these would be: rectal fluid 32%, urine 29%, and skin 39%. We do not have an accurate measure of urine  $\text{Na}^+$  so that we cannot partition the  $\text{Na}^+$  efflux. It is noteworthy that urine  $\text{Na}^+$  must be less than 3  $\mu\text{eq/ml}$ , assuming a rate of urine production of 75  $\mu\text{l}/10$  g-hr and rectal fluid excretion of 22  $\mu\text{l}/10$  g-hr and the concentrations indicated in Tables I and IV.

In PW earthworms remain in a steady state with respect to  $\text{Na}^+$  and  $\text{Cl}^-$  because they can transport each ion from the bath to the body fluids against an electrochemical gradient. For  $\text{Na}^+$ , at least, this capacity resides primarily in the integument since animals with their mouth and anus blocked have an influx of  $\text{Na}^+$  equal to or greater than normal animals. We have not studied  $\text{Cl}^-$  transport in this respect.

The influx of  $\text{Na}^+$  is a function of external  $[\text{Na}^+]$  at lower concentrations ( $< 3 \text{ mM Na/l}$ ) but at higher concentrations appears to be Na-independent (Fig. 3). Since the diffusive component of the influx is negligible over this concentration range, the magnitude of the influx suggests that the membrane permeability to  $\text{Na}^+$  is low. The curve relating  $M_i$  to external  $[\text{Na}^+]$  approximates a rectangular hyperbola which can be expressed by an equation analogous to the Michaelis-Menten equation for enzyme kinetics where  $V$  corresponds to influx:

$$I_r = \frac{V_{\max}[\text{Na}^+]}{K_s + [\text{Na}^+]} \quad (2)$$

For the earthworm adapted to PW and tested at various concentrations of  $\text{Na}^+$ ,  $V_m = 1 \mu\text{eq}/10$  g-hr and  $K_s = 1.3 \text{ mM/l}$ . The  $V_m$  value is comparable to values of some other fresh-water animals, for example the crayfish, 1.5 (Shaw, 1963), and the salamander, 3.0 (Alvarado and Dietz, 1970). The  $K_s$  of earthworms is higher than for most freshwater invertebrates but lower than marine forms. For example  $K_s$  for the fresh-water crayfish *Astacus* is 0.2  $\text{mM Na}^+/\text{l}$  whereas for *Carcinus*, a brackish-water crab, it is 20  $\text{mM Na}^+/\text{l}$  (Potts and Parry, 1964).

Compared to brackish water and marine annelids, earthworms exchange  $\text{Na}^+$  and  $\text{Cl}^-$  at a much lower rate. For example *Nereis* in dilute sea water (9‰) exchanges  $\text{Na}^+$  at about  $80 \mu\text{eq}/10 \text{ g-hr}$  ( $12^\circ \text{C}$ ) and  $\text{Cl}^-$  at about  $160 \mu\text{eq}/10 \text{ g-hr}$  ( $24^\circ \text{C}$ ) (Fretter, 1955; Jørgensen and Dales, 1957). Even when transferred to freshwater, *Nereis* has an influx of  $\text{Cl}^-$  of  $55 \mu\text{eq}/10 \text{ g-hr}$  (Jørgensen and Dales, 1957). Adaptation to freshwater in this Phylum has involved a marked reduction in the rate of exchange of ions between the animals and the bath.

The influx of  $\text{Na}^+$  and  $\text{Cl}^-$  is also dependent on the internal concentration of these ions. The influx for worms salt-depleted 3–4 days and placed in PW is elevated nine times for  $\text{Na}^+$  and 2.6 times for  $\text{Cl}^-$ . This response is characteristic of most fresh-water animals. The mechanisms are not known but a neuroendocrine reflex may be involved (see below).

Active transport of  $\text{Na}^+$  and  $\text{Cl}^-$  are largely independent processes. Sodium can be accumulated from  $\text{Na}_2\text{SO}_4$  without the concomitant uptake of  $\text{SO}_4^{2-}$ . This probably involves exchange with an endogenous cation such as  $\text{NH}_4^+$  or  $\text{H}^+$ . Chloride can be accumulated from  $\text{KCl}$  probably in exchange for endogenous  $\text{HCO}_3^-$ . We have not analyzed these mechanisms but the rates of excretion of  $\text{NH}_4^+$  and  $\text{HCO}_3^-$  are commensurate with the hypothesis.

When worms are transferred from soil to PW, ion and water balance are temporarily upset. Water is taken up and the animals gain weight for about two days but finally stabilize at about 115% of their weight in soil. The net uptake of  $\text{H}_2\text{O}$  initially is about  $92 \mu\text{l}/10 \text{ g-hr}$  which is a minimum estimate of the osmotic uptake of water under these conditions (Fig. 2).

Apparently the nephridia cannot immediately adjust to the increased uptake of water and the animals gain water. Inulin clearance in at least some (4 out of 10) soil-acclimated animals placed in PW was only  $10 \mu\text{l}/10 \text{ g-hr}$  or about eight times lower than PW adapted animals. Water balance is achieved when the osmotic force is balanced by hydrostatic force generated by the elasticity of the body wall plus the forces involved in eliminating water in urine and rectal fluid.

The net accumulation of water when transferred from soil to PW dilutes the solutes in the body fluids and activates the  $\text{Na}^+$  and  $\text{Cl}^-$  pumps. In effect the animal behaves as if it is salt-depleted. This leads to a positive  $\text{Na}^+$  and  $\text{Cl}^-$  balance and ultimately the concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in CF are restored to values almost identical with soil-animals. By varying the activity of the ion pumps rather than water content, these animals are able to adjust their ionic composition. This may reflect a direct effect of specific ions in the body fluids on exposed epithelia participating in transport or may involve a more elaborate neuroendocrine reflex mechanism analogous to what is found in vertebrates. Some evidence for the latter hypothesis has been provided by Kamemoto (1964) who reported a "brain factor" involved in water and salt balance in earthworms.

#### SUMMARY

1. Earthworms maintain a hyperionic steady state while living in a dilute solution containing  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{+2}$ ,  $\text{Cl}^-$  and  $\text{HCO}_3^-$  (PW).
2. The inside (coelomic fluid) of worms is electronegative by 12 to 30 mV relative to the bath (PW).

3. Sodium and chloride are transported across the skin against an electrochemical gradient. Each ion may be transported independently of the other presumably in exchange for an endogenous ion of like charge.

4. For animals in PW,  $\text{Cl}^-$  is exchanged at a rate of  $0.9 \mu\text{eq}/10 \text{ g-hr}$  and  $\text{Na}^+$  at a rate of  $0.3 \mu\text{eq}/10 \text{ g-hr}$ . The influx of  $\text{Na}^+$  is dependent on  $\text{Na}^+$  concentration in the bath and displays saturation kinetics ( $V_{\text{max}} = 1 \mu\text{eq}/10 \text{ g-hr}$ ;  $K_s = 1.3 \text{ mMoles/l}$ ). Chloride kinetics were not analyzed.

5. Salt depletion increases the influx of  $\text{Na}^+$  and  $\text{Cl}^-$  causing a net uptake of each ion.

6. Inulin and dextran are cleared from the coelomic fluid of PW-adapted worms at a rate of  $75 \mu\text{l}/10 \text{ g-hr}$ . Excretion of these compounds is probably through nephridia so that this is a maximum estimate of the rate of urine production. Water is also lost as a dilute rectal fluid which is formed at a rate of about  $20 \mu\text{l}/10 \text{ g-hr}$ .

7. Drinking of the bath occurs at a slow rate ( $4 \mu\text{l}/10 \text{ g-hr}$ ). The gut does not play a significant role in ion uptake from the bath but may be involved in the elimination of water. Possible mechanisms are discussed.

8. When worms are transferred from moist soil to PW they experience a net uptake and retention of water which dilutes their body fluids. Subsequently  $\text{Na}^+$  and  $\text{Cl}^-$  influxes are elevated resulting in the net accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  and, within seven days, the restoration of the original (soil) concentrations in the coelomic fluid ( $75 \text{ mM Na}^+/\text{l}$ ,  $47 \text{ mM Cl}^-/\text{l}$ ).

9. Several lines of evidence indicate that hydromineral metabolism in worms is delicately controlled and involves the integrated activity of several organs. These control systems are discussed.

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